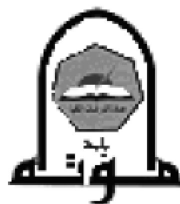


Mu’tah University
Deanship of Graduate Studies

Comparative Study on the Activity and Kinetic of Polyphenol Oxidase Enzyme Extracted From Different Plant Sources

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Dedication

To my father ,
My mother,
My brothers and sisters.

Nooh M. Al- Khamaisa

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List of Abbreviations

PPO	Polyphenol oxidase
1/V	1/ Velocity
1/[S]	1/ substrate concentration
V_{max}	Maximum reaction velocity
K_m	Michaelis-Menten constant
SD	Standard deviation
AE	Activation energy

Abstract

Comparative Study on The Activity and Kinetic of Polyphenol Oxidase Enzyme Extracted From Different Plant Sources.

Nooh M. Al-Khamaisa

Mu'tah University, 2013

Polyphenol oxidases (PPOs) from *Pyrus malus* (apple), *Cucumis sativus* (cucumber), *Cucurbita pepo* (cucurbita), *Punica granatum* (pomegranate) seed, *Punica granatum* (pomegranate) rind and *Opuntia ficus-indica* (cactus) were studied. Crude PPO extracts from these plants were characterized in terms of pH, temperature, enzyme kinetic and effects of some chemical agents on its activity. The enzymatic browning was quantified by spectrophotometer for 100 min. The PPO activity was tested by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 410 nm. Apple showed the highest browning intensity when compared to the tested plants and pomegranate rind showed the lowest . The highest activity for PPO using catechol as substrate was found in apple (V_{max}= 0.333 µmol /min) and the lowest in pomegranate rind (V_{max}= 0.03 µmol /min). High correlation was found between the enzymatic browning and PPO activity which supports the hypothesis that PPO was responsible for browning of fruits. The PPOs exhibited the maximum activity at pH 7 for apple, cucumber, cactus and cucurbita, and at pH 6 for pomegranate rind and pomegranate seed. cactus and cucumber showed two optimum pH values (4 and 7) which might have resulted from the presence of isoenzymes. The optimum temperature for PPO was ranging from 35 to 40°C. Most of PPOs extracted from these plants have very low activation energy . The study of the effects of some chemical agents on PPO activity indicated that urea was the most potent inhibitor for PPOs while proline acted as an activator for the PPOs in all of tested plants.

الملخص

دراسة مقارنة لنشاطية و حركية أنزيم البولي فينول أوكسيديز المستخلص من مصادر نباتية مختلفة.

نوح الخمايسة

جامعة مؤته، 2013

تمت دراسة أنزيمات البولي فينول أوكسيديز (PPO) المستخلصة من ثمار التفاح، ثمار الخيار، ثمار الكوسا، بذور الرمان، قشر الرمان، و أوراق الصبر. الدراسة الحالية استخدمت المستخلص الخام لأنزيم PPO من هذه النباتات لتشخيص كل من تأثير درجة الرقم الهيدروجيني، تأثير درجة الحرارة، حركية الأنزيم و تأثير بعض العوامل الكيميائية على نشاطية الأنزيم. تم تحديد قيم التصبغ البني من خلال جهاز المطياف الضوئي خلال 100 دقيقة. تم استخدام المطياف الضوئي لقياس نشاطية الأنزيم من خلال قياس المعدل الابتدائي لتكون الكينون كما يشير لذلك الارتفاع في قيمة الامتصاصية الضوئية. أظهر التفاح أعلى قيمة تصبغ بني مقارنة بالنباتات الأخرى ، بينما أظهر قشر الرمان أقل قيمة. وجدت أعلى نشاطية لأنزيم PPO باستخدام الكاتيكول كمادة متفاعلة في نبات التفاح (السرعة القصوى للتفاعل = 0.333 ميكرومول/دقيقة) و أقل نشاطية في قشور الرمان (السرعة القصوىللتفاعل = 0.03 ميكرومول/دقيقة) . وجد أن هناك ارتباط عالي بين التصبغ البني و نشاطية الأنزيم مما يدعم فرضية مسؤولية هذا الأنزيم عن التصبغ البني في الثمار. أظهر الأنزيم أعلى نشاطية عند رقم هيدروجيني 7 في كل من التفاح، الخيار، الصبر و الكوسا ، و عند رقم هيدروجيني 6 في كل من قشور و بذور الرمان . أظهر كل من الصبر و الخيار وجود رقمين هيدروجينيين مثليين هما 4 و 7 و الذي قد يكون ناتجا عن وجود نظائر الأنزيم. كانت درجة الحرارة المثلى للأنزيم تتراوح من 35-40مْ. معظم أنزيمات PPO المستخلصة من هذه النباتات كانت تمتلك طاقة تنشيط منخفضة جدا. أظهرت دراسة تأثير العوامل الكيميائية على نشاط الأنزيم أن اليوريا كانت أقوى مثبط للأنزيم ، بينما كان البرولين منشطا للأنزيم في جميع نباتات الدراسة .

Chapter One
Theoretical Background

1.1 Introduction.

Polyphenol oxidases (PPOs) are oxidoreductases belonging to a group of copper containing metalloproteins that catalyze oxidation of phenolic compounds by utilizing molecular oxygen (Queiroz *et al*., 2008) . These enzymes are widely distributed among microorganisms, plants and animals. According to their substrate specificities and mechanism of actions, PPOs are subdivided into three subclasses, tyrosinases (E.C. 1.14.18.1), catechol oxidase (E.C. 1.10.3.1) and laccase (E.C. 1.10.3.2). All of these groups catalyze the o-hydroxylation of monophenols to o-diphenols . They can also further catalyze the oxidation of o-diphenols to produce o-quinones in the presence of atmospheric oxygen (Duran *et al*., 2000). The rapid polymerization of o-quinones to produce black, brown or red pigments is the cause of fruit browning (Mayer, 2006).

In plants, PPOs are located mainly in thylakoid membrane of chloroplasts and mitochondria (Liu, 2005). Approximately, 50% of vegetables and fruits are discarded due to quality defects resulting from enzymatic browning by PPOs (Fujita *et al.*, 2006). Because of the bad effect of enzymatic browning on food products, PPO has been extensively studied in a variety of plant tissues (Marshall *et al*., 2000).

Due to their ability to act on phenolic compounds, PPOs are considered as highly useful biocatalysts for various biotechnological applications (Simsek and Yemenicioglu, 2007). In the food industry, browning is a serious detriment occurring during processing and storage, especially during manufacture of fruit, vegetable and meat product. Brown discoloration decreases the quality of food products due to changes in color, nutritional, sensory and flavor properties (Quesnel and Jugmohunsingh, 2006).

PPOs are associated with plant defense, upon tissue damage by mechanical injury, feeding by herbivores or insects and infection by pathogens, cellular compartmentalization is lost and PPO from plastids can react with phenolic substrates from the vacuole. Polyphenols are responsible for the darkening of tissue during lesion formation and are thought to seal off wounds or infected tissue to limit secondary infection or further spread of pathogens. Enzymes produced by pathogens are inactivated by quinones and plant proteins become unavailable for nutrition (Chen *et al*., 2005 ; Raj *et al*., 2006).

Extraction, purification and characterization of plant PPO had been focused on fruits and vegetables because of the significance of enzymatic browning in post-harvest physiology and food technology. To date, there are few studies reported on the isolation and characterization of PPO from *Pyrus malus* (apple), *Cucumis sativus* (cucumber), *Cucurbita pepo* (cucurbita)

and *Punica granatum* (pomegranate) and *Opuntia ficus-indica* (cactus). The present study was conducted to obtain information about PPO from the above plants, and also to describe some properties of the enzyme and the inhibitory effect of some substances.

1.2 Aims of the Study

1. To determine the relationship between time and browning intensities in the following plants: *Pyrus malus* (apple) fruit, *Cucumis sativus* (cucumber) fruit, *Cucurbita pepo* (cucurbita) fruit, *Punica granatum* (pomegranate) Seed, *Punica granatum* (pomegranate) rind, and *Opuntia ficus-indica* (cactus) leaf.
2. To determine the activity and kinetics (K_m and V_{max}) of PPO enzyme in the above plants.
3. To investigate the influence of pH, temperature on PPO activity.
4. To investigate the effect of some chemical agents on PPO activity.

Chapter Two
Literature Review

2.1 Phenolics in Plants

Plants are considered as rich sources of phenolic compounds, which can act as antioxidant, antimicrobial, anti-inflammatory and antidiabetic agents. The protection afforded by the consumption of plant products like vegetables, fruits and legumes is associated with the presence of phenolic compounds (Jin and Mumber, 2010).

Phenolic compounds in plant are classified as simple phenols or polyphenols based on the number of phenol units in the molecule, they are synthesized partly as a response to physiological and ecological pressures like insect attack (Wijngaard *et al*., 2009).

The basic structural feature of phenolic compounds is an aromatic ring consists of one or more hydroxyl groups. The plant phenolic comprise simple phenols, such as coumarins, tannins, phenolic acids and flavonoids (Figure 1) (Jin and Mumber, 2010). Flavonoids are one of the most common phenolic, distributed in plant tissues, and responsible alongside the carotenoids and chlorophylls for their blue, purple, yellow, orange and red colors. The family of flavonoids includes flavones, iso-flavonols, flavonols, anthocyanins, proanthocyanidins, anthocyanidins and catechins (Stalikas, 2007).

Another major class of phenolic compounds is the cell wall phenolics. They are insoluble and found in complexes with other types of cell components. The two main groups of cell wall phenolics are lignins and hydroxycinnamic acids (Stalikas, 2007). These compounds play a critical role in the cell wall during plant growth by protecting against stresses such as infection, wounding and UV radiation (Zhang *et al*., 2011).

2.2 Polyphenol Oxidases

Polyphenol oxidases (PPOs) (EC 1.14.18.1 or EC 1.10.3.2) are ubiquitous plant enzymes that catalyze the O_2-dependent oxidation of mono and diphenols to o-quinones. These quinones are reactive species that are able to covalently modify and cross-link a wide variety of cellular nucleophiles via a 1,4 addition mechanism, leading to the formation of polymeric brown or black pigments, which are responsible for significant post harvest losses of fruits and vegetables (Vámos-Vigyázó, 1981).

A mix of catechol oxidase and monophenol oxidase enzymes are found in nearly all plant tissues, and can also be found in fungi, bacteria and animals. In insects, cuticular PPOs are present and their products are responsible for desiccation tolerance (Vichapong, *et al.*, 2010).

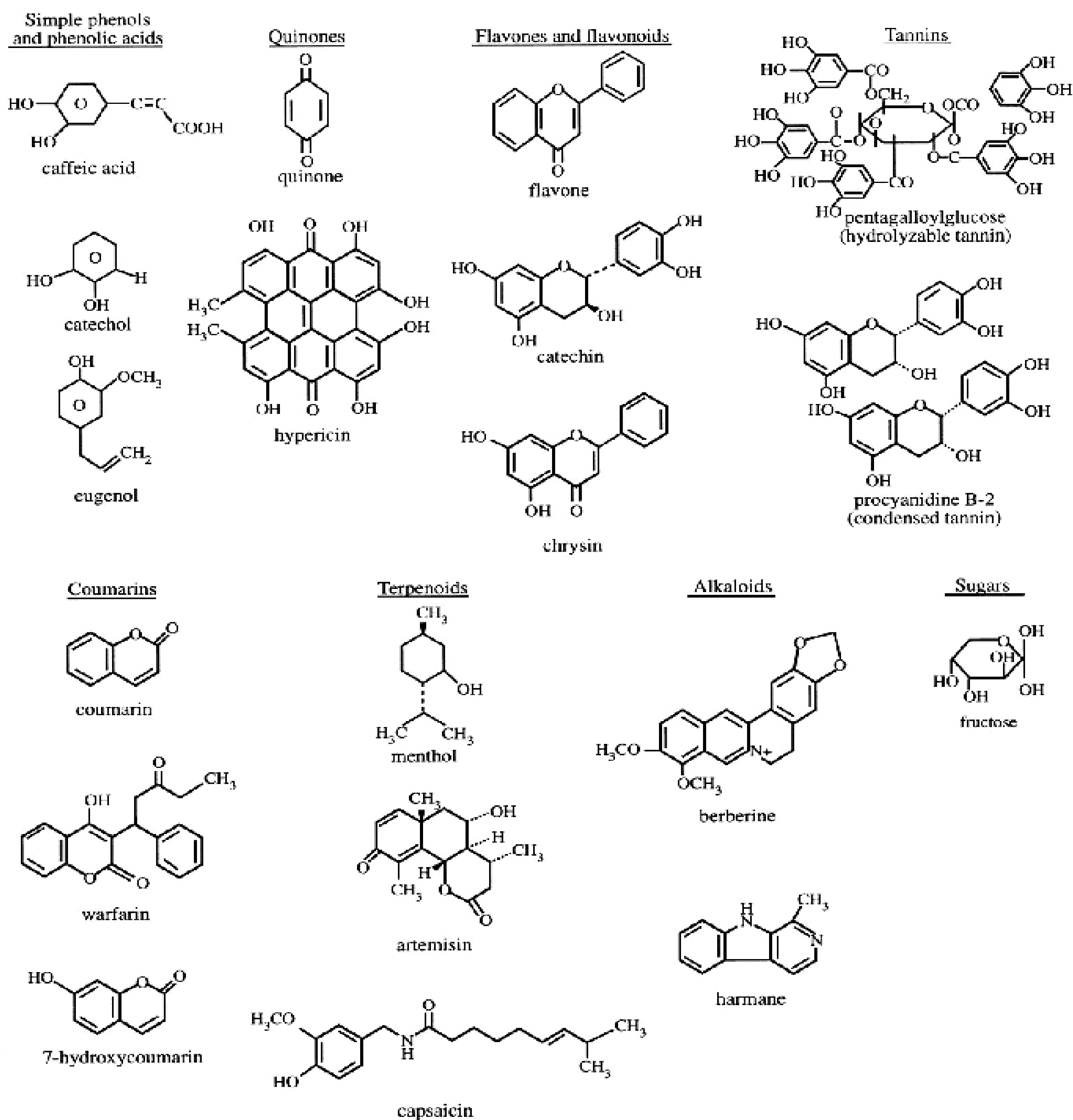
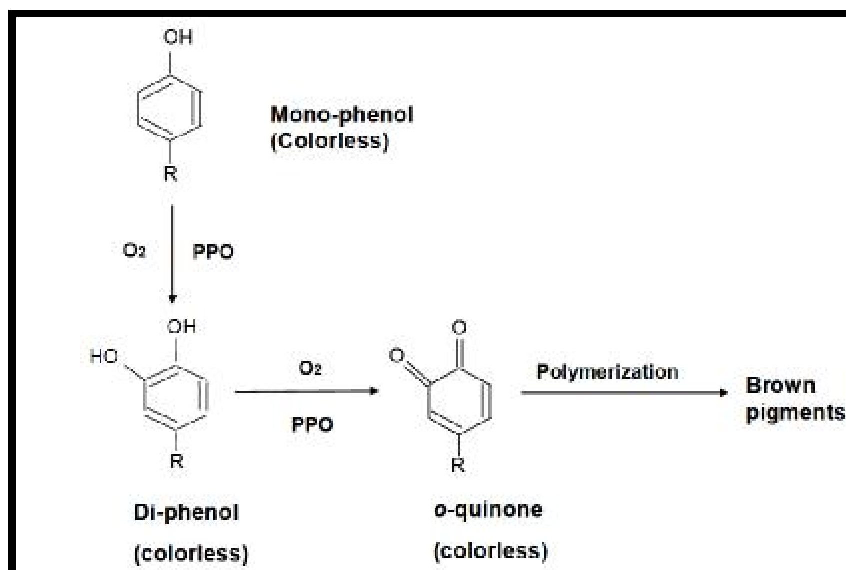


Figure (1)
Basic structures of phenol and flavonoid compounds (Jin and Mumber, 2010).

2.2.1 Properties of PPOs

PPO is a copper (Cu^{2+}) containing metalloenzyme predominantly located in the chloroplast thylakoid membrane (Anthon and Barrett, 2002). The two atoms of Cu^{2+} are tightly ligated to three histidine residues of a polypeptide chain (Klabunde *et al*., 1998). PPO exists in three different types of isoforms namely oxy-PPO [Cu(II) Cu(II) O_2], met-PPO [Cu(II) Cu(II)] and deoxy-PPO [Cu(I) Cu(I)] (Likhitwitayawuid, 2008) and they have very diverse and variable molecular weights e.g., *Eriobotrya japonica* Lindl: 59.2-61.2 kDa (Sellés-Marchart *et al*., 2006), *Phaseolus vulgaris* L: 120 kDa (Beena and Gowda, 2000), *Malpighia glabra* L : 52 and 38 kDa (Kumar *et al*., 2008), and *Brassica rapa* **:** 65 kDa (Nagai and Suzuki, 2001).

PPO catalyzes two reactions including hydroxylation of monophenols to give *o*-diphenol (monophenol oxidase, cresolase tyrosinase activity EC. 1.14.18.1) (Fawzy, 2005) and oxidation of *o*-diphenol to *o*-quinones (diphenol oxidase, catechol oxidase activity EC.1.10.3.1) (Mayer, 2006; Madani *et al*., 2011). The *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments. Chlorogenic acid, catechin and epicatechin are considered as a naturally occurring PPO substrates in fruits and vegetables (Fig. 2) (Queiroz *et al*., 2008).



Figure(2)
Schematic representation of oxidation reaction by PPO(Queiroz, *et, al*., 2008).

2.2.2 Occurrence and Location of PPOs

Polyphenol oxidases are widely distributed in nature, they can be found in almost all living organisms. Tyrosinase is mostly found in animals including humans, and it is involved in the pigmentation of skin, hair and eye by melanin synthesis. In insects, polyphenol oxidases are responsible for the exoskeleton formation (Halaouli*, et al.*, 2006).

In plants, Leaves and fruits of many plant species have been the major target tissues to investigate the occurrence of PPOs. Tyrosinase activity has important roles in metabolism of plants including wound healing system (Kong, *et al.*, 2000).

In fungi, polyphenol oxidases, particularly laccases, play role in lignin degradation, fungal spore formation and pigmentation, detoxification of toxic compounds, pathogenesis (fungal virulence factors) and fungal morphogenesis (Ruijssenaars and Hartmans, 2004).

The function of bacterial polyphenol oxidases is not fully understood because a little attention has been paid to study these enzymes in these organisms. The best documented function of polyphenol oxidase in bacteria is the formation of melanins which are polyphenolic pigments, protect the bacterial spores and cells against oxidants, free radicals and UV radiation (Claus and Decker, 2006; Dalfard *et al.*, 2006).

2.2.3 Industrial and Environmental Applications of Polyphenol Oxidases

PPOs have high capacity for oxidizing aromatic compounds. This feature makes the use of polyphenol oxidases very suitable for some biotechnological applications in food industry, pulp and paper industry, textile industry, medicine and environmental technology. PPOs were used in food industry for the improvement of flavor in tea, cocoa, coffee production, ascorbic acid determination, sugar beet pectin gelation and as a biosensor (Polaina and MacCabe, 1974).

In industrial preparation of paper, PPOs are used in the removal of lignin from woody tissues and pulp bleaching , they can be even used for deinking ,decolorizing a printed paper (Guebitz and Cavaco, 2003 ; Couto and Herrera., 2006).

In environmental technology, PPOs can be used in the development of biosensors for immunoassays, for the detection and removal of phenols and phenolic compounds from wastewater (Duran and Esposito, 2000). Peroxidases and polyphenol oxidases are used as an enzymatic approach for the removal of phenolics and the toxic compounds from wastewater before they are discharged into the environment from industrial effluents such as coal conversion, petroleum refining, wood preservation, textile, paper, food and chemical industries (Sukan and Sargin, 2013).

PPOs have the ability to treat phenolic compounds over wide ranges of pH and temperature. In this aspect polyphenol oxidases appear to be more

advantageous because they require only molecular oxygen as oxidant to work. PPO has been used as an insecticide and antimicrobial fumigant in dried fruits, nuts, and spices in the United States for over fifty years (United States Environmental Protection Agency, 2006).

In medical applications, PPOs were used to inhibit the adhesion of some bacteria (Cowan *et al*., 2000), for treatment of Parkinson's disease (Xu *et al.*, 1998) and used as a prodrug therapy agent and as a tumor-suppressing (Seo, *et al*., 2003).

2.3 Food Browning

Generally, food browning results from several different processes that fall into two categories : non-enzymatic reactions and enzymatic oxidation of phenolic and other compounds (Rodriguez and Toca, 2006) .

2.3.1 Non-Enzymatic Browning

Non-enzymatic browning is a chemical process that changes the color of food to brown, and it occurs without enzymes being involved. There are three main types of non-enzymatic browning: Maillard reaction, Ascorbic acid oxidation, and Caramelization (Simsek and Yemenicioglu, 2007).

The most common type of non-enzymatic browning is the Maillard reaction which involves a series of chemical reactions that occur when amino acids and reducing sugars are heated together. The sugar interacts with amino acids, producing various aromas and imparting color to foods. The type of amine and reducing sugar influences the reaction rate as well as the products formed, which ultimately are brown melanoidin pigments (Duran and Esposito, 2000)

Ascorbic acid oxidation causes the loss of vitamin C from food. This browning is the spontaneous thermal decomposition of ascorbic acid under both aerobic and anaerobic conditions. It was reported that over 80% of the browning of dried apple during storage packed under vacuum resulted from oxidative by non-enzymatic reaction (Rodriguez and Toca, 2006).

Carmelization is the process in which sugar is oxidized by heat, this process is used extensively in the food industry for generating nutty flavor and brown color. Volatile chemicals are released during the reactions and produce the caramel flavor. The brown color of caramel is used in a variety of beverages, such as soft drinks (Pereira *et al*., 2009).

2.3.2 Enzymatic Browning

Enzymatic browning is one of the most important reactions that occur in fruits and vegetables, usually resulting in negative effects on color, taste, flavor, and nutritional value. The reaction is a consequence of phenolic compounds oxidation by PPO, which triggers the generation of dark pigments. This is particularly relevant for apples, which are rich in

polyphenols and highly susceptible to enzymatic browning (Holderbaum *et al.*, 2010). Enzymatic browning has a role in the browning discoloration by reducing the shelf life of many processed foods, and affecting the production of dehydrated and frozen fruits and vegetables (Haard and Chism, 1996). Simsek and Yemenicioglu (2007) reported that enzymatic browning is a desired reaction in processes like tea and cocoa fermentation and in the formation of raisin, date and prune products.

2.4 Polyphenol Oxidase in Plants

There are mainly three types of polyphenol oxidases in plants, classified according to their substrate specificities and mechanism of actions. These are; tyrosinase, catechol oxidase and laccase (Hernandez-Romero *et al*., 2006). PPO has been extensively studied and characterized in a variety of tissues, as potato and taro (Duangmal and Arslan, 1999); egg plant , pears (Gauillard and Richard-Forget, 1997); grapes (Rapeanu *et al*., 2006); tea leaves (Halder *et al*., 1998); peppermint (Kavrayan and Aydemir, 2001); oregano (Dogan *et al*., 2005) and artichoke (Aydemir, 2004).

Several studies have shown that the enzymatic browning in apple pulp is associated with polyphenol content and/or PPO activity (Nicolas *et al*., 2007), in a study for Coseteng and Lee (2006) about the changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning, they found that polyphenoloxidase (PPO) activity and polyphenol concentrations decreased during maturation and remained relatively constant during cold storage.

Silva *et al*., 2005 reported that the fruits of cucurbita are considered as a rich source of enzymes as peroxidase and polyphenol oxidases. These enzymes have applications in various areas such as, pharmaceutical industry, food technology and process biochemistry.

In pomegranate (*Punica granatum*) , little information are known about PPO activity. In a study for Jaiswal *et al.*, 2008, anthocyanins have been found to be heat stable and results suggest that PPO is the primary enzyme responsible for oxidative degradation of anthocyanins in dried arils.

Low molecular phenolics derived from benzoic acid (gallic acid, vanillic acid, 3,4-dihydroxy- benzoic acid, ethyl 3,4-dihydroxybenzoate) and from cinnamic acid (sinapic acid, ferulic acid, p-coumaric acid) were found in fruits (Chang *et al*., 2008) and cladodes (Qiu *et al*., 2003) of cactus (*Opuntia spp.*). Some of them are known as precursors in the biosynthesis of more complex secondary metabolites (Luckner 1990). Important enzymes involved in the transformation of these phenolic substances are polyphenol oxidases (Mayer, 2006). Since a PPO from *O. dillenii* cladodes shows pH optima at 4.0, 7.0 and 10.0, it should consist of three isoenzymes, the activity of which was reduced by several acids in the following order: ethylene diamine tetraacetic acid < ascorbic acid < citric acid (Qin *et al*., 2004).

2.5 Polyphenol Oxidase Inhibitors

Prevention of enzymatic browning is a major challenge for vegetable and fruit processing in food industry. PPO is the enzyme responsible for this deteriorative process. Enzymatic reaction rate can be decreased by a group of substances called inhibitors, the function of these inhibitors is to scavenge oxygen, shift reducing potential, and reduce oxidation (Casado-Vela *et al*., 2006). Tropolone, potassium pyrosulphite, sulfur dioxide, citric acid, ascorbic acid and erythorbic acid chemicals widely used for browning inhibition and used as antioxidant in food industry because of their antioxidant properties (Kim and Uyama, 2005).

Chapter three
Materials and Methods

3.1. Plant Materials

Pyrus malus (apple) fruit, *Cucumis sativus* (cucumber) fruit, *Cucurbita pepo* (cucurbita**)** fruit, *Punica granatum* (pomegranate) seed, *Punica granatum* rind, *and Opuntia ficus-indica* (cactus) leaf were collected from Yarout region in south Jordan in September, 2012 (Table 1).

Table (1)
The botanical data of the studied plants.

Scientific name	Common name (English)	Common name (Arabic)	Parts used
Pyrus malus	Apple	التفاح	Fruit
Cucumis sativus	Cucumber	الخيار	Fruit
Punica granatum	Pomegranate	الرمان	Fruit (Seed and rind)
Opuntia ficus-indica	Cactus	الصبار	Leaves
Cucurbita pepo	Cucurbita	الكوسا	Fruit

3.2. Chemicals

1. Sodium fluoride (CODEX LTD Carloerba, Milan,Italy).
2. Catechol (BDH laboratory Supplies, poole,England).
3. L-tyrosine(BDH laboratory Supplies, poole,England).
4. Thioourea (BDH laboratory Supplies, poole,England).
5. Di-sodiume hydrogen phosphate anhydrous (Fluka-Chemieka AGCH9470 Buchy).
6. Sodium-dihydrogen phosphate (Panreac, Montplet & Esteban SA, Barcelona-Espana).
7. Sodium acetate (Riedel-Dehaen.Sigma-Aldrich Laborchemilealien GmbH Seelze).

3.3. Crud Enzyme Preparation

Fresh fruits or leaves (50 grams) of each plant were homogenized with the addition of sodium fluoride solution (1:5 w/v) in a blender for 3 min. The homogenate was filtered through several filter papers, and then was

centrifuged for 10 min at low speed. The supernatant was collected as crude enzyme solution and was kept at 4^{o}C (Atrooz, 2009).

3.4. Browning Intensities Measurement:-

For each sample, a cuvette was filled with 1.5 ml of freshly prepared crude enzyme solution , and the optical density (absorbance) was measured at wavelength 410 nm by a spectrophotometer (spectrophotometer UV/ Vis Biotech Engineering Management co. LTD, UK) at intervals of 20 min for 100 min. The absorbance was taken as the browning intensity and relative percentage (%) of browning intensity was calculated (Atrooz, 2009).

3.5. Assay of PPO Activity and the Effects of Substrate Concentration.

PPO activity was determined by measuring the increase in absorbance at 410 nm with a spectrophotometer. The substrate (catechol) was assayed at different concentrations : 0.01 M, 0.03M, and 0.05 M at pH 7.0 and temperature 40^{o}C. The sample tube contained 2.0 ml of desired concentration of catechol, 0.9 ml of 0.2 M sodium phosphate buffer (pH 7.0) and 0.1 ml of enzyme solution. The reference tube (blank) contained 2.0 ml of the same substrate solution and 1.0 ml of 0.2 M sodium phosphate buffer. One unit of PPO activity was defined as the change in absorbance of 0.001 min^{-1}(Mustafa *et al*., 2007). Data were plotted as (1/velocity) and (1/substrate concentration) according to Lineweaver & Burk's (1934) method of plotting. Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) of the enzyme PPO were determined from the plot (Mustafa *et al*., 2007).

3.6. Effect of pH on PPO Activity

The effect of pH on enzyme activity was investigated using acetate and phosphate buffers at different pH values. The optimum pH of the enzyme was determined under the standard assay conditions by measuring activity in the presence of buffers at different pH values ranging from 3.5 to 8.0 (Mizobutsi *et al*., 2010). Reaction rates of the enzymes will be assayed as described by Mustafa *et al*., 2007 and relative activity (%) was calculated .

3.7. Effect of Temperature on PPO Activity

The effect of temperature on polyphenol oxidase activity was examined under standart assay conditions at different temperatures ranging from 25 to 55 ^{o}C and the buffer was heated to relevant temperature before the assay (Segel, 1976). Reaction rates of these enzymes was assayed as described by Mustafa *et al*., 2007 and relative activity (%) was calculated .

An Arrhenius plot of log of enzyme activity (Log V) versus reciprocal of absolute temperature (K^{-1}) was constructed to determine the activation energy (AE) of the enzyme (Ibrahim *et al*., 2012).

3.8. Effect of Some Chemical Agents on PPO Activity

The reaction mixture contained 2.0 ml of 20 mM catechol, 0.4 ml of 0.2 M sodium phosphate buffer (pH 7.0), and 0.5 ml of chemical agent (Proline, Tyrosine and Urea) solution in 0.2 M sodium phosphate buffer (pH 7.0) and 0.1 ml of enzyme solution. Each of the inhibitors was assayed at various concentrations (0.1mM, 1.0mM and 10mM) (Güray, 2009).

3.9 Statistical Analysis

All data analysis were performed using the Microsoft excel 2003. All experiments were done in triplicates .Results were reported as mean± standard deviation(SD).

Chapter Four
Results, Discussions and Recommendations

4.1 Characterization of PPO

4.1.1 Browning Measurement

Enzymatic browning is a widespread color reaction occurring in fruits and vegetables and tea leaves. The browning reaction requires the presence of oxygen, phenolic compounds and PPO, and is usually initiated by the enzymatic oxidation of monophenols into o-diphenols, and o-diphenols into quinones, which undergo further non-enzymatic polymerization leading to the formation of pigments. Although enzymatic browning is beneficial to the color and flavor development of certain food items such as tea, coffee and cocoa, it impairs the quality and salability of fresh-cut produce. A variety of fruits and vegetables, such as lettuce, potato, apple, pear, banana and peach, are susceptible to enzymatic browning during processing and storage (Nicolas *et al.*, 2007).

This study showed a relationship between the browning intensity and time as shown in figure 3. The highest browning intensity was found in apple, followed by pomegranate seed and cucumber. The browning intensity in all tested plants was found to increase initially and became constant after about 80 min. These results could be explained by the level of PPO activity and concentration of phenolic compounds that can vary between varieties of plants . Furthermore, a tissue's PPO level can vary depending on growing conditions and tissue maturity. These results are in agreement with the findings of Atrooz, (2009) and Nguyen *et al.*, (2003) who suggested the PPO activity as the main factor in the browning reaction.

4.1.2 Effect of pH on PPO Activity

The effect of pH on PPO activity was investigated by measuring enzyme activity at different pH values ranging from 3.5 to 8 . The pH profile of PPO was shown in Figure (4). The optimum activity of the enzyme was seen at pH 7 for apple, cucumber, cactus and cucurbita, and at pH 6 for pomegranate rind and pomegranate seed , The PPO of cactus leaf and cucumber showed two peaks at pH 7 and at pH 4 . The relative activity of PPO at pH 4 was 74% for cucumber and 94% for cactus (figure 5).

In many plants, PPO enzyme has only one optimum pH, such as in Potato it was 5.0 (Balasingam and Ferdinand, 1970), Medlar fruits 6.5 (Barbaros *et al.*, 2002), Longan fruit 7.0 (Jiang, 1999), but the possession of two optimum pH values might have resulted from the presence of isoenzymes . This result is supported by the fact that PPOs from some other plants have two pH optima, such as cherry tomatoes (Saeidian, 2012) and sweet cherry (Pifferi and Cultrera, 1974). Generally the optimum pH for PPO in the most of plants lies between 6.0 to 7.0 (Bello *et al.*, 2011 ; Mizobutsi *et al.*, 2010)

with lower activity levels occurring at more acidic or alkaline pH values, depending on extraction methods, substrate used and the localization of the enzyme in the plant cell (Aylward and Haisman, 1969) .

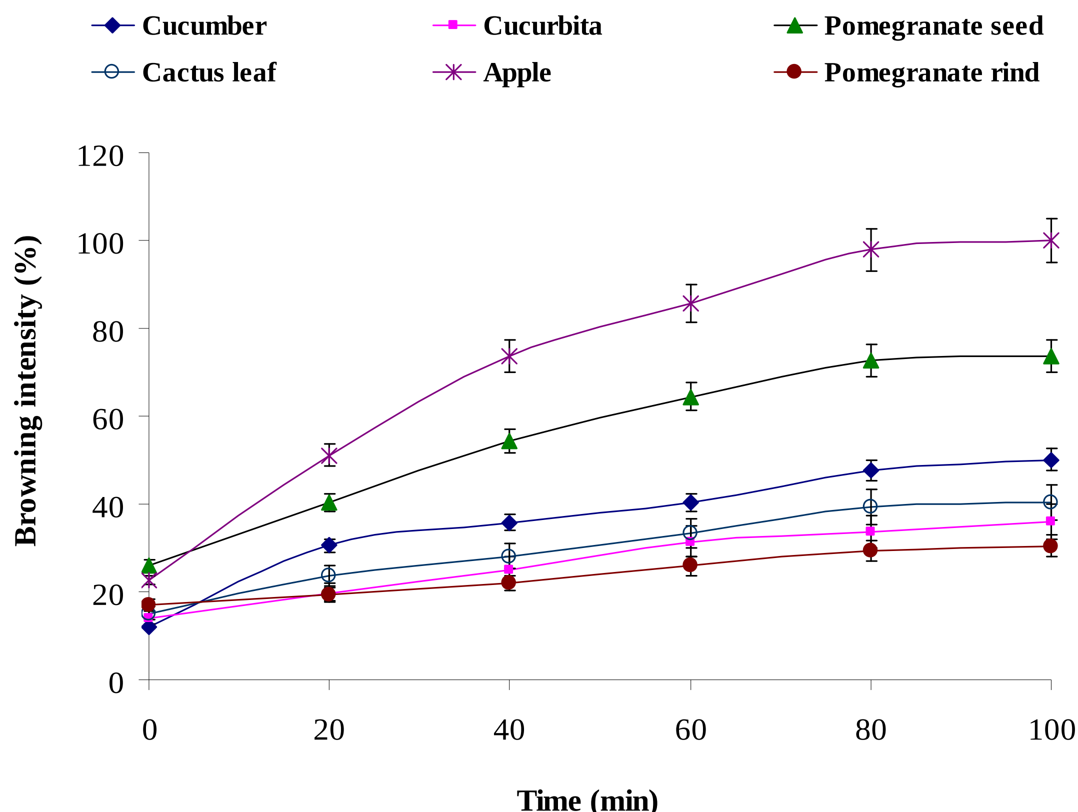


Figure (3)
Browning intensity (%) of apple fruit, pomegranate rind, pomegranate seed , cactus leaf, cucumber fruit and cucurbita fruit with time (min). Mean±SD, n=3.

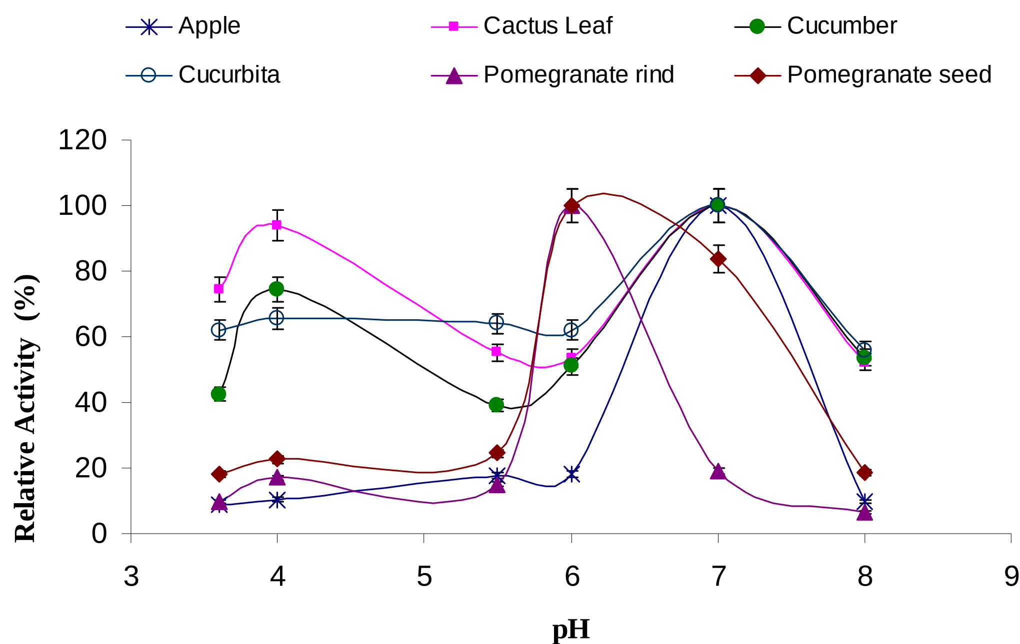


Figure (4)
Relative activity (%) of PPO in apple, cucurbita, pomegranate rind , pomegranate seed, cactus leaf and cucumber at different pH values. Mean±SD, n=3.

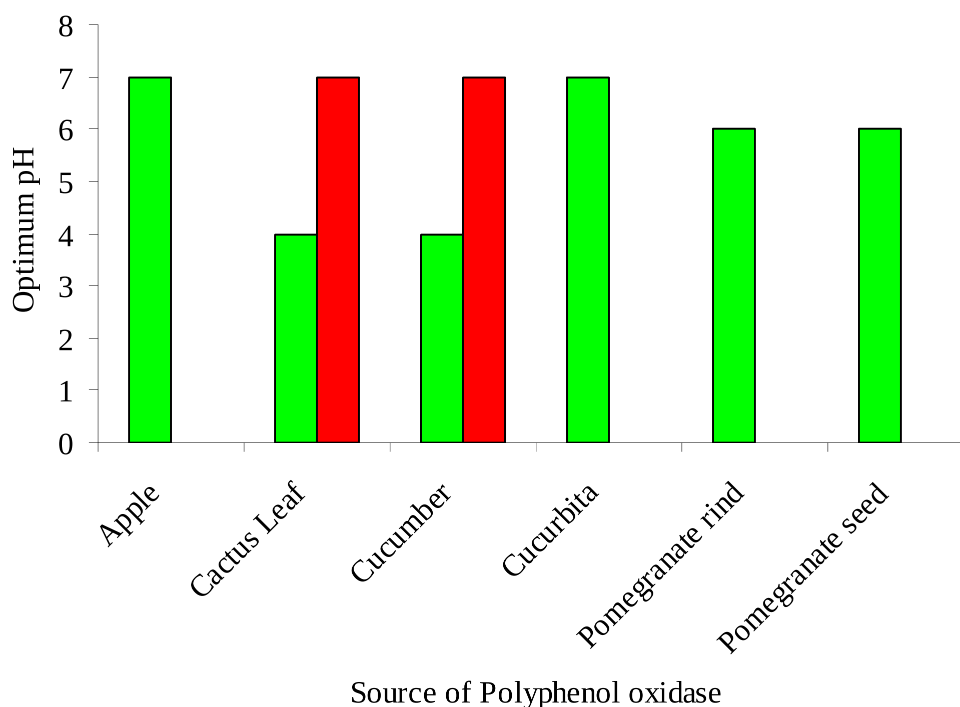


Figure (5)
Optimum pH values for PPO in apple, cucurbita, pomegranate rind , pomegranate seed, cactus leaf and cucumber .

4.1.3 Effect of Temperature on PPO Activity

The effect of temperature on PPO activity is shown in figure 6. The optimal temperature found for PPO activity in these plants was ranging from 35 to 40ºC . At 30^{o}C, the relative activity was ranging from 45% in cactus leaf to 72% in cucumber. Raising the temperature above 45ºC caused the activity to decline drastically which may be due to the effect of denaturation . The same optimum temperature was reported for thyme (40ºC) (Dogan *et al.*, 2006) , Medler fruits (35^{o}C) and Anna apple (35^{o}C), but lower optimum temperature was reported for Bayberry (30ºC) (Fang, 2008) , Stanly plum (25ºC) (Siddig *et al.*, 1992) and grape (25-30ºC)(Mustafa *et al.*, 2007).

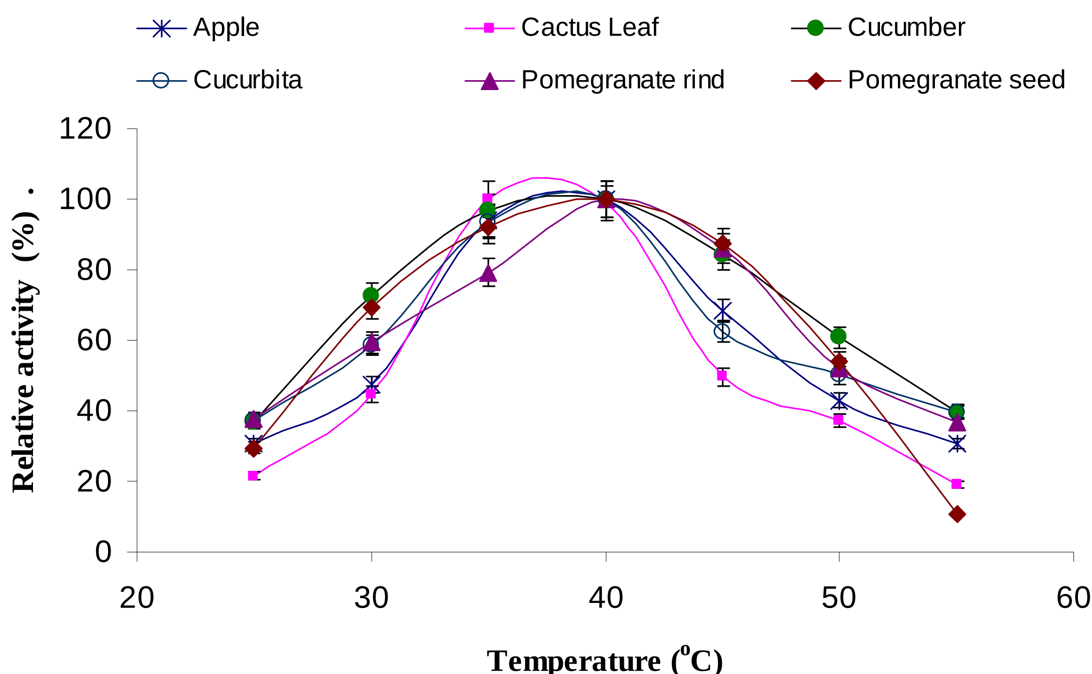


Figure (6)

Relative activity (%) of PPO in apple, cucurbita, pomegranate rind , pomegranate seed, cactus leaf and cucumber at different temperatures . Mean±SD, n=3.

Results of this study and previous studies clearly demonstrate the sensitivity of PPO to heat, the activity can therefore be controlled by inducing either low or high temperatures because the low temperature inhibit the activity and the high temperature causes enzyme denaturation.

Determination of optimum temperature is an important factor for the selection of enzymes for industrial applications. Interestingly, Devi *et al.*, (2007) reported that most industrial enzymes have V_{max} at 40-50^{o}C . The studied plants have optimum temperature closed to 40^{o}C that means they can be used in industrial applications.

The Arrhenius plots of the PPO of apple, cucumber, cucurbita, cactus leaf, pomegranate rind and pomegranate seed gave an AE values of 1.724, 2.639, 0.5455, 3.617, 0.7431 and 10.795 KJ/mol respectively .

Moreover, it is thus clear from figure (7) that most of PPOs extracted from these plants have very low AE which is useful and required in the industrial processes to form the product.

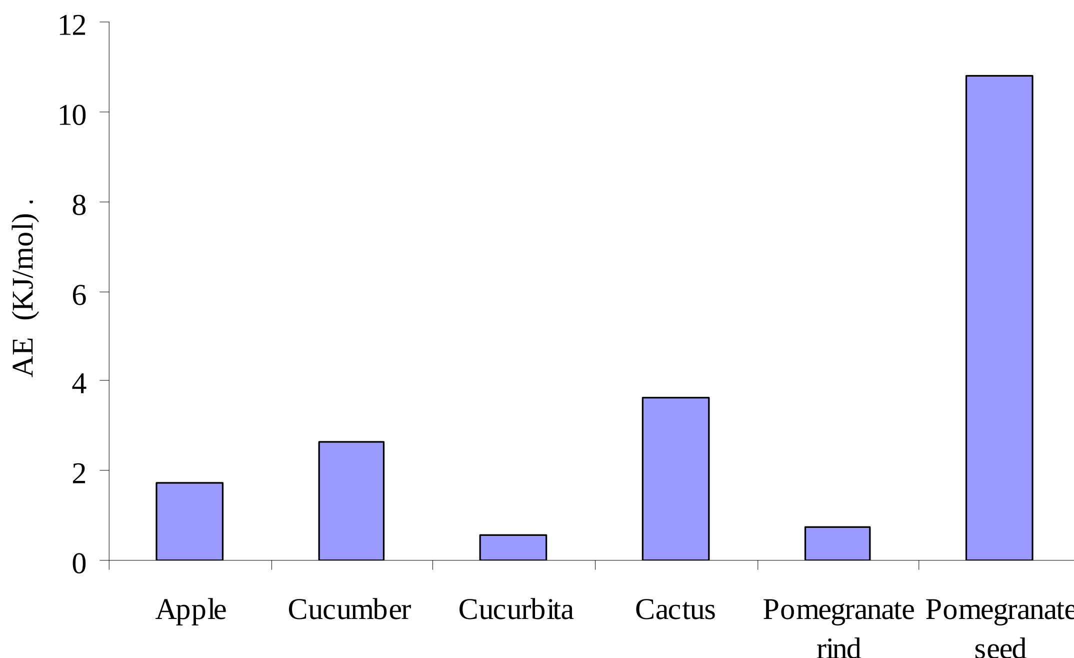


Figure (7)

Activation energy (AE) for PPOs extracted from apple, cucumber, cucurbita, cactus leaf, pomegranate rind and pomegranate seed.

4.1.4 Kinetic Analysis

To determine the kinetic constants, K_m and V_{max} of PPO, initial reaction rates at different catechol concentrations (0, 0.025, 0.0375 and 0.050 M) were measured. In order to obtain Lineweaver-Burk plot; 1/V values were plotted against 1/[S] values (Figure 8) and kinetic constants were calculated from the graph. The results of K_m and V_{max} values of the enzyme were summarized in table 2.

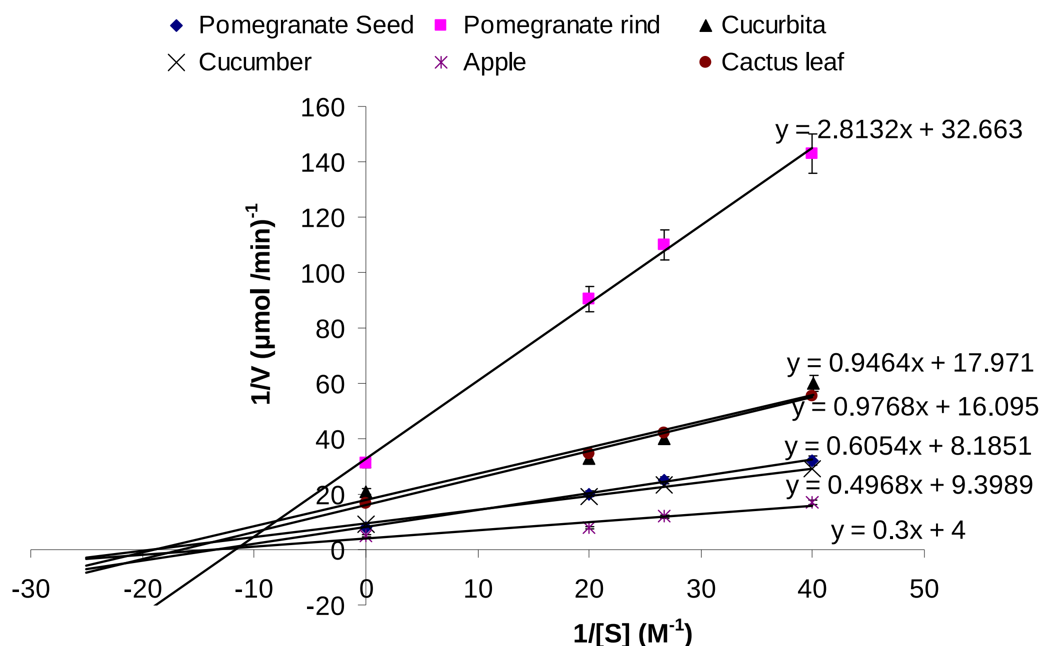


Figure (8)

Determination of K_m and V_{max} values for PPOs from different plant sources, using catechol as substrate. Mean±SD, n=3.

Table (2)

Kinetic values for PPOs from different plant sources using catechol as substrate at 40°C and pH 7.

Source of PPO	**V_{max}** (µmol /min)	**K_m** (M)
Cactus Leaf	0.062	0.058
Apple	0.189	0.067
Cucumber	0.106	0.053
Cucurbita	0.056	0.05
Pomegranate rind	0.03	0.083
Pomegranate Seed	0.122	0.083

The highest activity (V_{max}) of PPO was seen in apple (0.333 µmol /min) , while the pomegranate rind has the lowest (0.03 µmol /min). these results are in accordance with the results of browning intensity experiment in which the apple exhibited the highest browning intensity. Such results suggests the PPO activity as the main factor in the browning reaction.

Figure (9) shows the ratio of V_{max}/K_m for the tested plants and from this ratio we can determine the affinity of the enzyme to the substrate. apple had

the highest V_{max}/K_m ratio, which means that the PPO of apple has the highest affinity for catechol as a substrate. The order of the affinity was : apple > cucumber > pomegranate seed > cactus leaf > cucurbita > pomegranate rind.

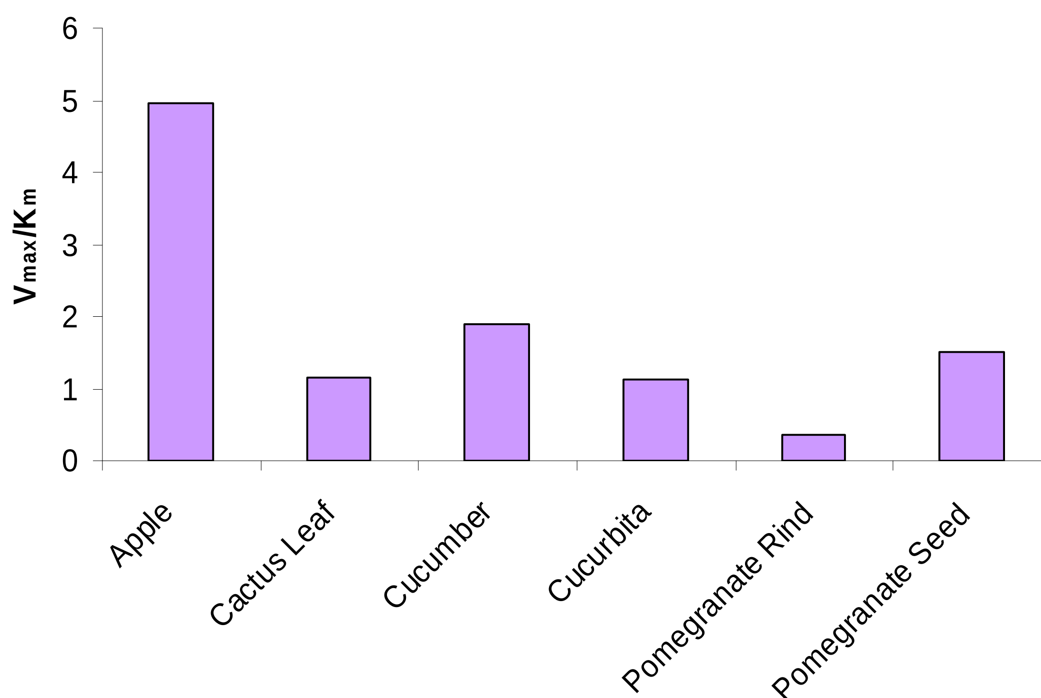


Figure (9)
The ratio of V_{max}/K_m for the tested plants

4.1.5 Correlations of Browning intensity with PPO activity

A strong correlation between the browning intensity and PPO activity in all plant samples was found (Correlation coefficient (R) = 0.9485). The linear correlation is shown in Figure (10).

These results are in agreement with the findings of Atrooz (2009) , Nguyen *et al*., (2003) and Cheng and Crisosto (1995) who suggested the PPO as one of the principal factors that affect browning in plants. These findings proved the hypothesis that PPO was responsible for brown discoloration of the fruit tissue when damaged or exposed to molecular oxygen during storage and processing. For a maximum storage stability and shelf-life, the fruits should be kept at sub-freezing temperatures.

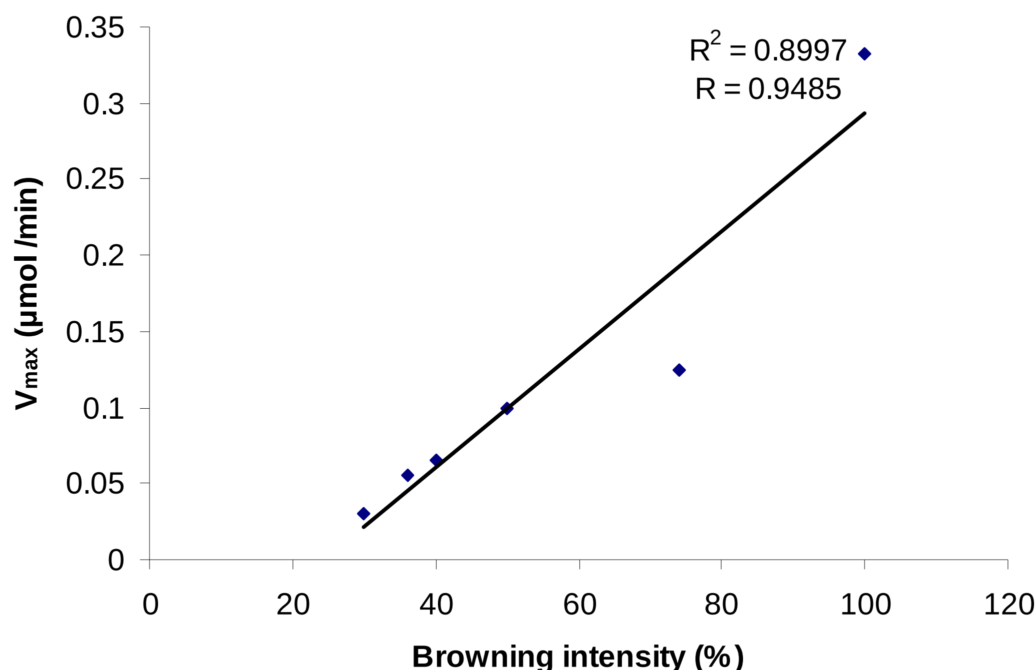


Figure (10)
The correlation between browning intensity(%) and PPO activity (µmol/min) .

4.1.6 Effect of Some Agents on PPO Activity

According to the literature, different effectors can control enzymatic browning and these compounds are classified based on the inhibition mechanism as reducing agents, chelating agents, acidulants, enzyme inhibitors, enzyme treatments and complexing agents (Özoglu and Bayiindirli, 2002) .

The most widespread agents used for browning control are sulphiting agents. Due to adverse health effects caused by these compounds, the World Health Organization (WHO) has recommended limiting, as much as possible, the use of SO_2 in the treatment of foodstuffs, even to the point of contemplating the possibility of its complete suppression. According to the current indications of WHO, the acceptable daily intake of SO_2 in foodstuffs has been established as 0.7 mg/kg of body weight. Several studies have been made to find an efficient inhibitor without toxic effects (Rapeanu *et al.*, 2006). For example Ascorbic acid was frequently used for browning control of food products (Özoglu and Bayiindirli, 2002).

The prevention of enzymatic browning by a specific inhibitor may involve a single or more mechanisms of inhibitor action. There are various mechanisms through which enzyme inhibitors can act which are competitive , uncompetitive and noncompetitive inhibition (Dogan *et al.*, 2005).

In the present study, the effect of three types of chemical agents (Proline, Tyrosine and Urea) on PPO Activity was investigated. The results which are shown in figures (11, 12, 13, 14, 15 and 16) and table (3) indicated that proline acted as an activator for the PPO in all of the tested plants , while tyrosine showed mild inhibitory effect on the enzyme (Noncompetitive-type with cactus and cucumber, and Uncompetitive-type with apple, cucurbita, pomegranate seed and pomegranate rind) , and a strong inhibitory effect was seen for urea (Noncompetitive-type with cucumber, and Uncompetitive-type with cactus, apple, cucurbita, pomegranate seed and pomegranate rind) .

These results are in agreement with the studies done by Saeidian (2012) and Yerlitürk, *et al*., (2008) who found that the presence of tyrosine and urea lead to the inhibition of the activity of PPO extracted from wild pears.

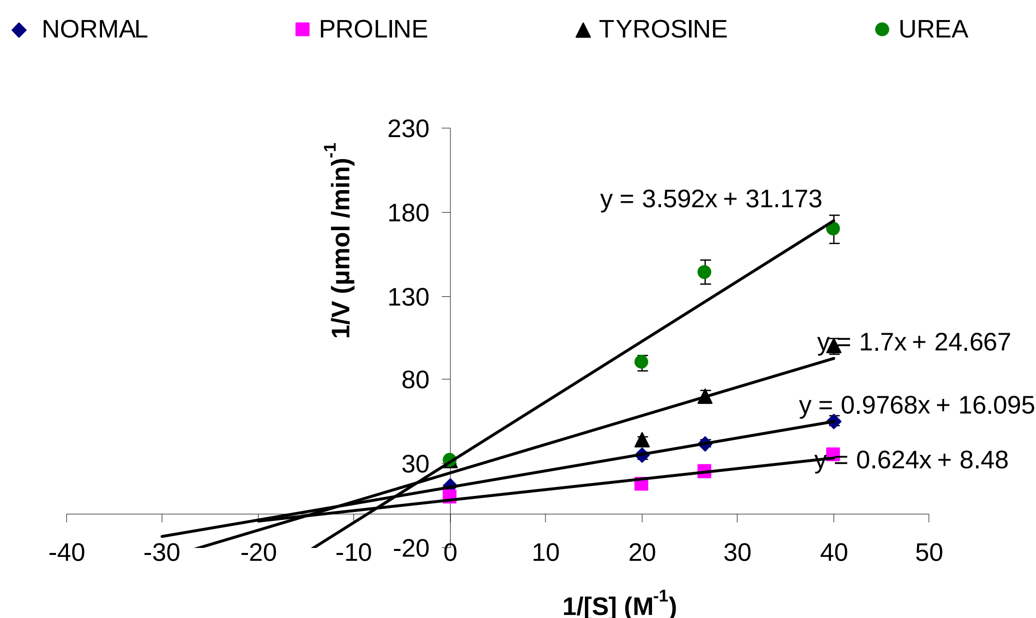


Figure (11)

Determination of K_m and V_{max} values for PPO from cactus leaves in the presence or absence of Proline, Tyrosine and Urea. Mean±SD, n=3.

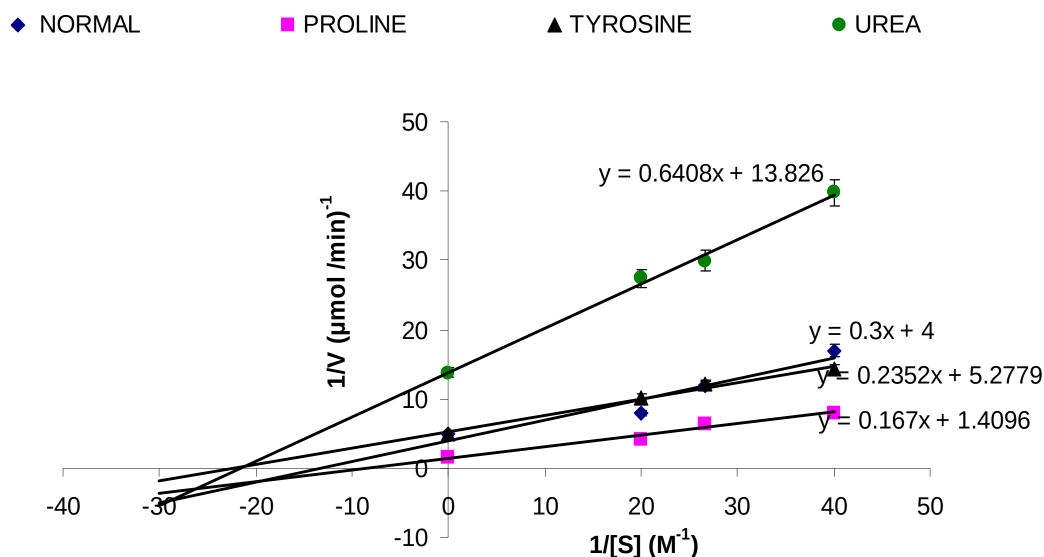


Figure (12)
Determination of K_m and V_{max} values for PPO from apple fruit in the presence or absence of Proline, Tyrosine and Urea.

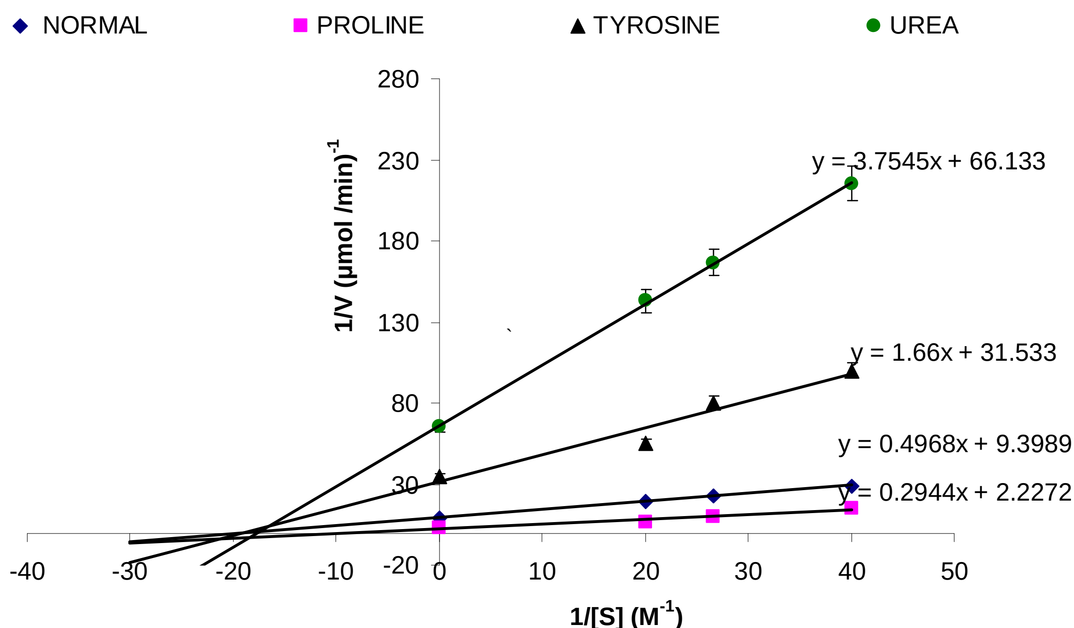


Figure (13)
Determination of K_m and V_{max} values for PPO from cucumber fruit in the presence or absence of Proline, Tyrosine and Urea. Mean±SD, n=3.

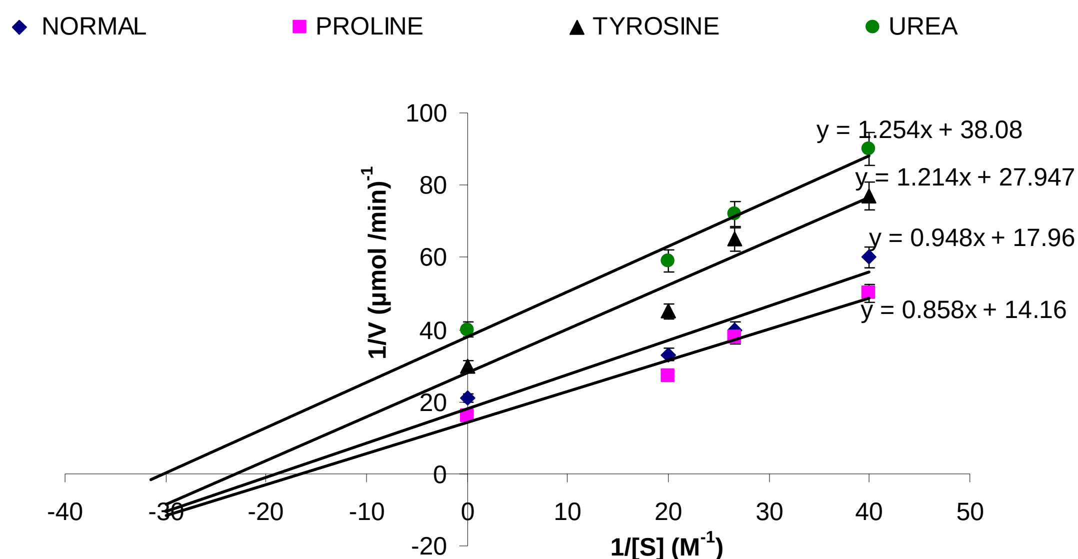


Figure (14)
Determination of K_m and V_{max} values for PPO from cucurbita fruit in the presence or absence of Proline, Tyrosine and Urea. Mean±SD, n=3.

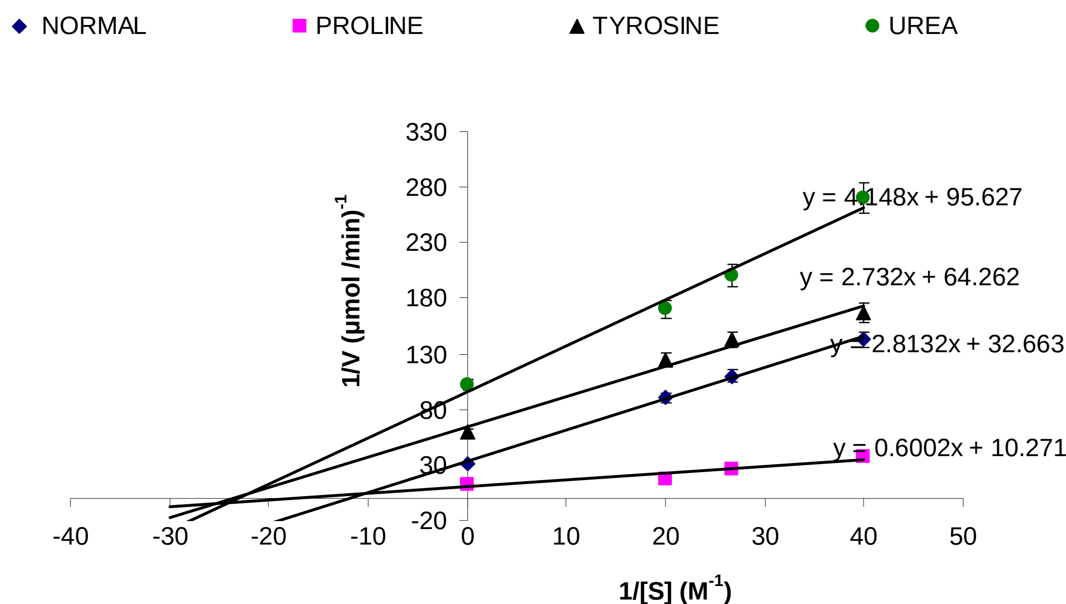


Figure (15)
Determination of K_m and V_{max} values for PPO from pomegranate rind in the presence or absence of Proline, Tyrosine and Urea. Mean±SD, n=3.

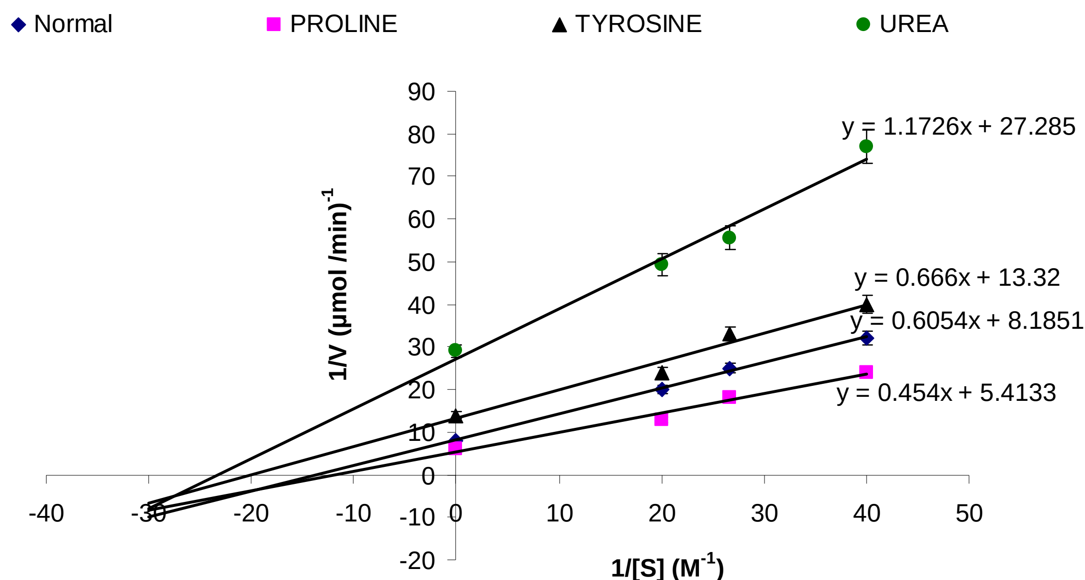


Figure (16)

Determination of K_m and V_{max} values for PPO from pomegranate seed in the presence or absence of Proline, Tyrosine and Urea. Mean±SD, n=3.

Table (3)

Kinetic values for PPO in the tested plants using catechol as substrate with and without some chemical agents .

Plant	Control		Effect of chemical agents					
			Proline		Tyrosine		Urea	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Cactus Leaf	0.062	0.058	0.117	0.071	0.04	0.058	0.032	0.125
Apple	0.189	0.067	0.7	0.111	0.25	0.048	0.072	0.048
Cucumber	0.106	0.053	0.449	0.125	0.031	0.053	0.015	0.053
Cucurbita	0.056	0.05	0.07	0.063	0.036	0.043	0.026	0.033
Pomegranate rind	0.03	0.083	0.097	0.067	0.015	0.04	0.011	0.04
Pomegranate Seed	0.122	0.083	0.185	0.083	0.075	0.05	0.037	0.045

4.2 Conclusions

1. This study showed apple fruit to have the highest browning intensity and PPO activity when compared to the tested plants .
2. The PPOs of apple, cucumber, cactus and cucurbita had its maximum activity at pH 7.0, while pomegranate rind and pomegranate seed at pH 6 (with catechol as substrate).
3. Two optimum pH values were found for PPO of cactus and cucumber (4 , 6 and 4 , 8 respectively) and this may indicate the presence of isoenzymes.
4. The optimum temperature for PPO in all of the tested plants was ranging from 35 to 40°C.
5. The study of the effects of some chemical agents on PPO activity indicated that urea was the most potent inhibitor for PPOs while tyrosine was moderate inhibitor and proline acted as an activator for the PPOs in all of the tested plants.

4.3 Recommendations

1. The PPO of these plants can be used for the development of biosensors to detect phenolic compounds for various purposes, so further studies are needed for this aim.
2. Further studies can be done to use these plants as sources of PPO to be used for the degradation of phenols in industrial wastewater to minimize the water pollution.

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appendix I

determination of K_m and V_{max} values for PPOs from different plant sources using Lineweaver–Burk plots

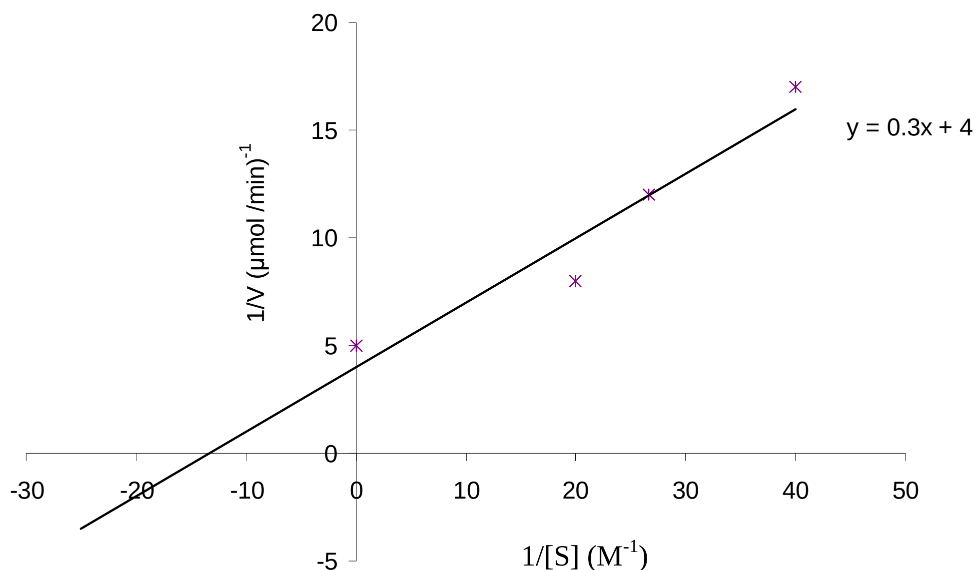


Figure (1)

Determination of K_m and V_{max} values for PPO from apple fruit by Lineweaver–Burk plot using catechol as substrate at 40°C and pH 7. Mean±SD, n=3.

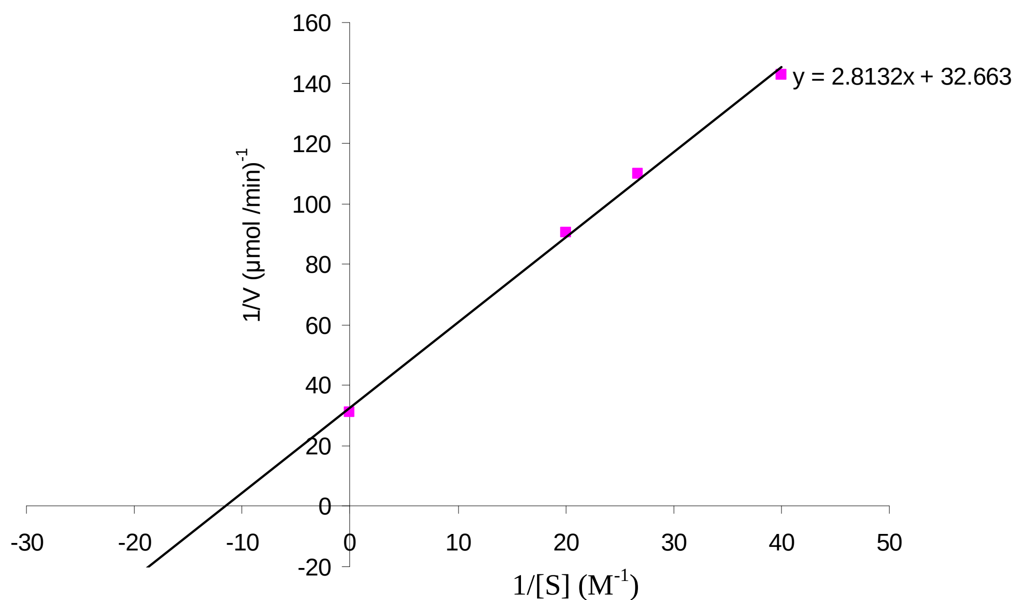


Figure (2)

Determination of K_m and V_{max} values for PPO from pomegranate rind by Lineweaver–Burk plot using catechol as substrate at 40°C and pH 7 . Mean±SD, n=3.

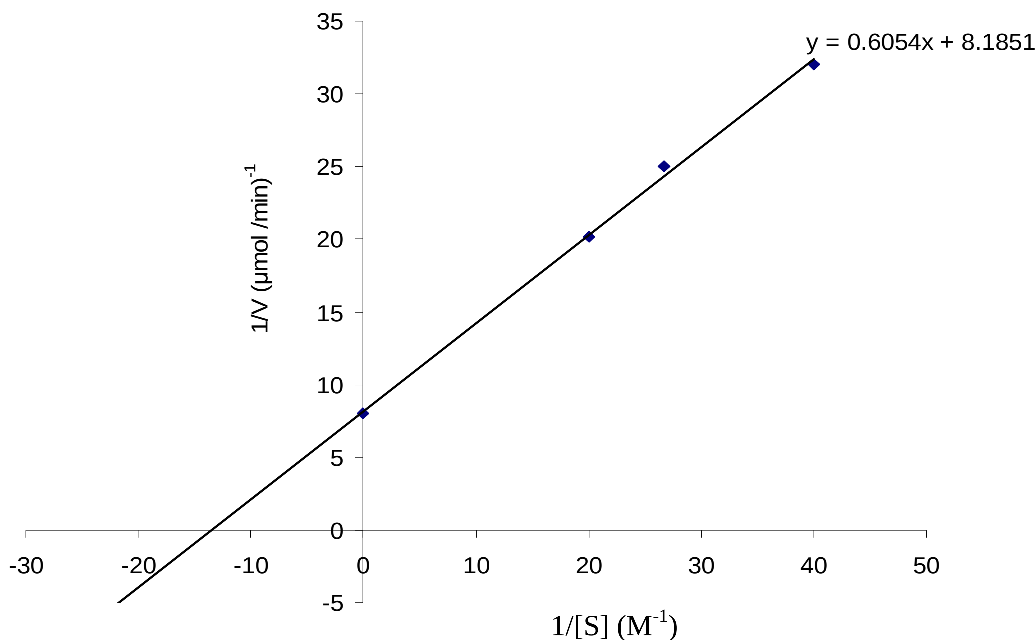


Figure (3)
Determination of K_m and V_{max} values for PPO from pomegranate seed by Lineweaver–Burk plot using catechol as substrate at 40^{o}C and pH 7. Mean±SD, n=3.

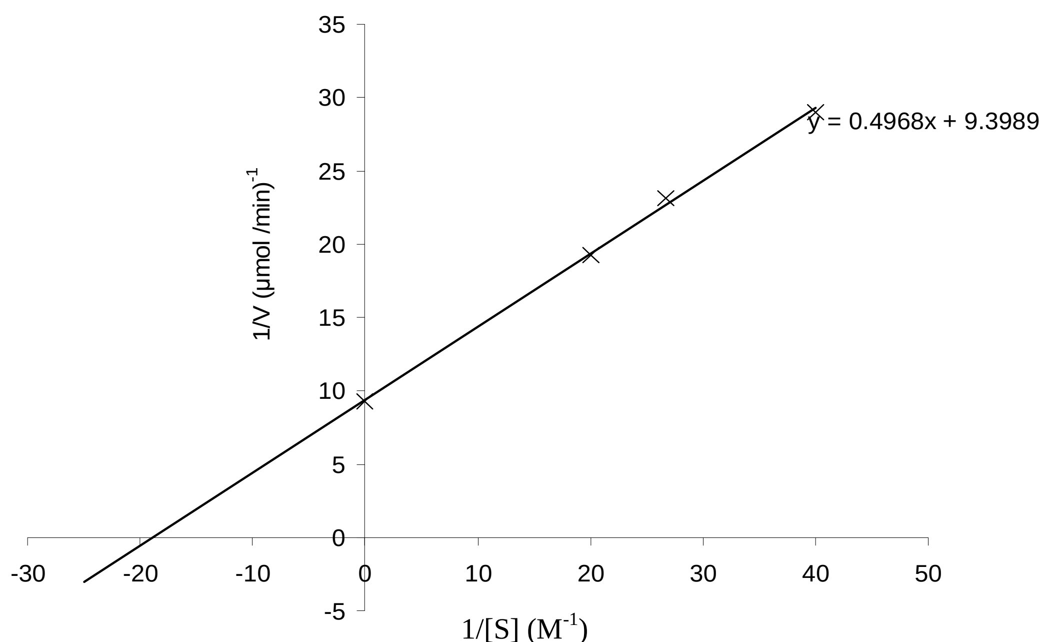


Figure (4)
Determination of K_m and V_{max} values for PPO from cucumber fruit by Lineweaver–Burk plot using catechol as substrate at 40^{o}C and pH 7. Mean±SD, n=3.

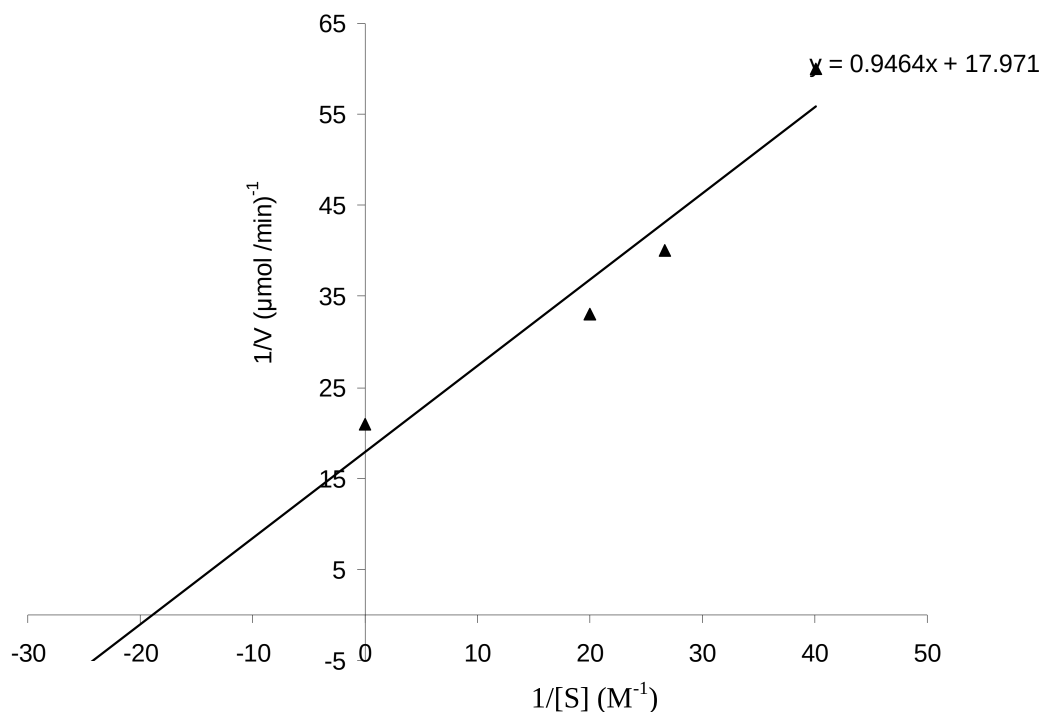


Figure (5)

Determination of K_m and V_{max} values for PPO from cucurbita fruit by Lineweaver–Burk plot using catechol as substrate at 40°C and pH 7. Mean±SD, n=3.

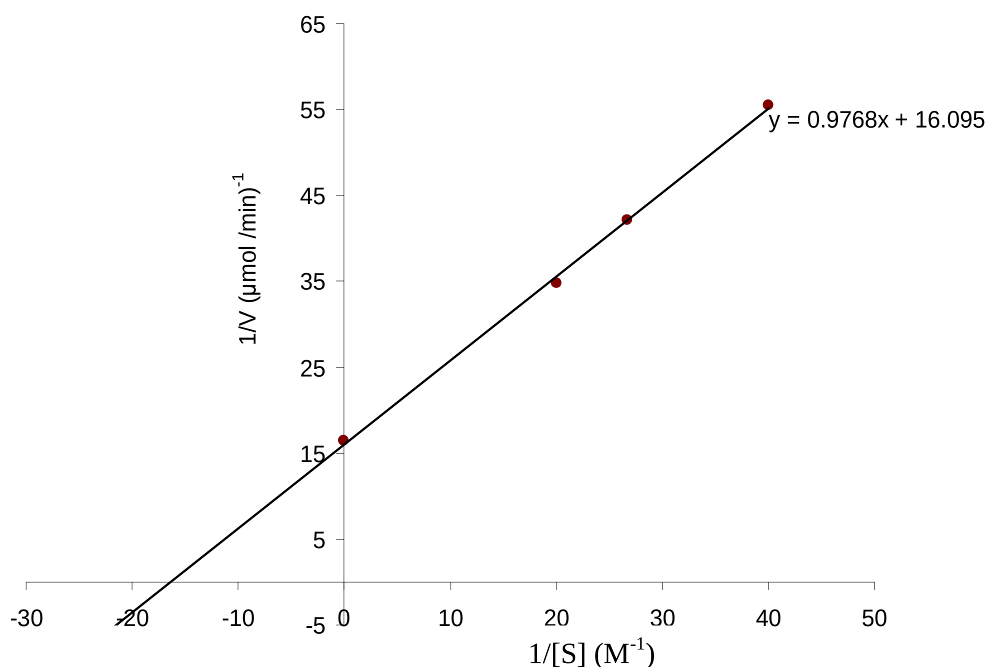


Figure (6)

Determination of K_m and V_{max} values for PPOs from cactus leaf by Lineweaver–Burk plot using catechol as substrate at 40°C and pH 7. Mean±SD, n=3.

appendix II

Arrhenious plot for the determination of activation energy for the tested plants.

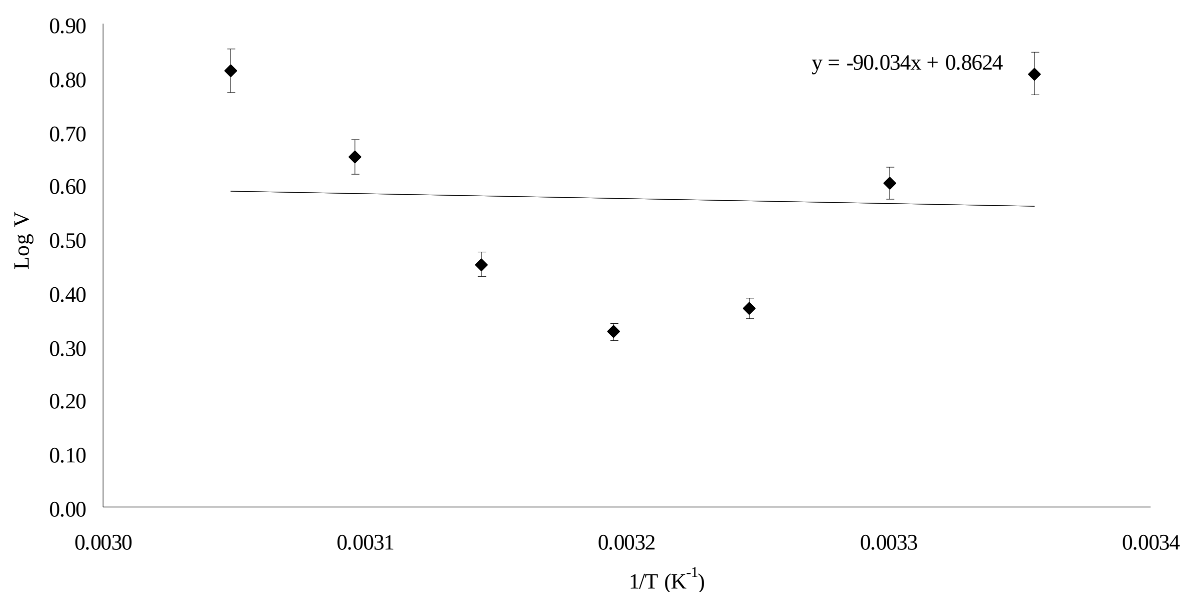


Figure (1)
Arrhenious plot for the determination of activation energy for PPO of apple. Mean±SD, n=3.

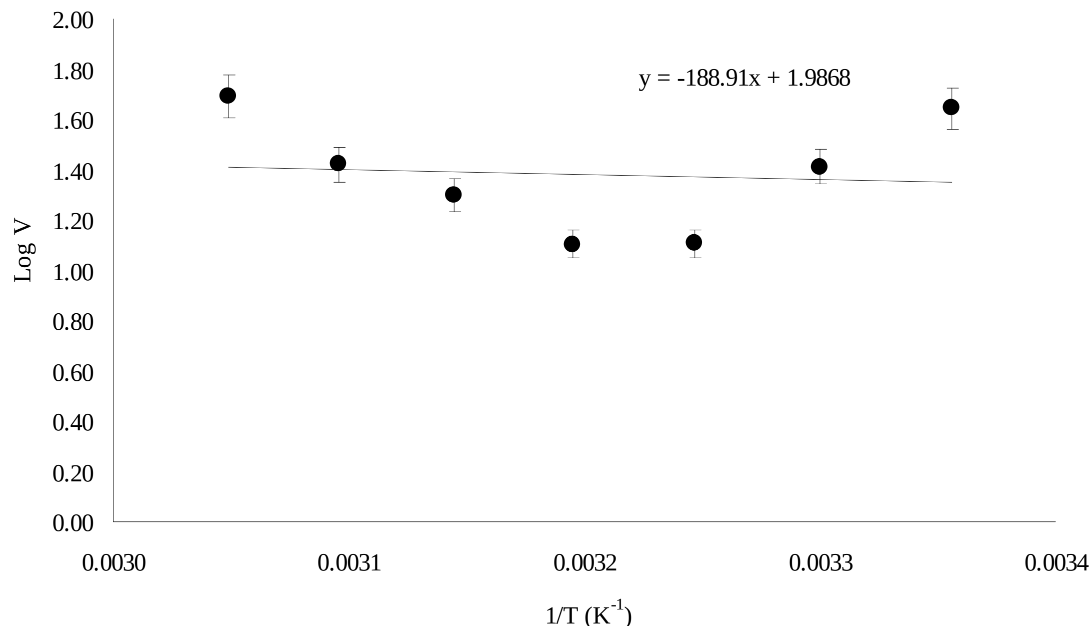


Figure (2)
Arrhenious plot for the determination of activation energy for PPO of cactus. Mean±SD, n=3.

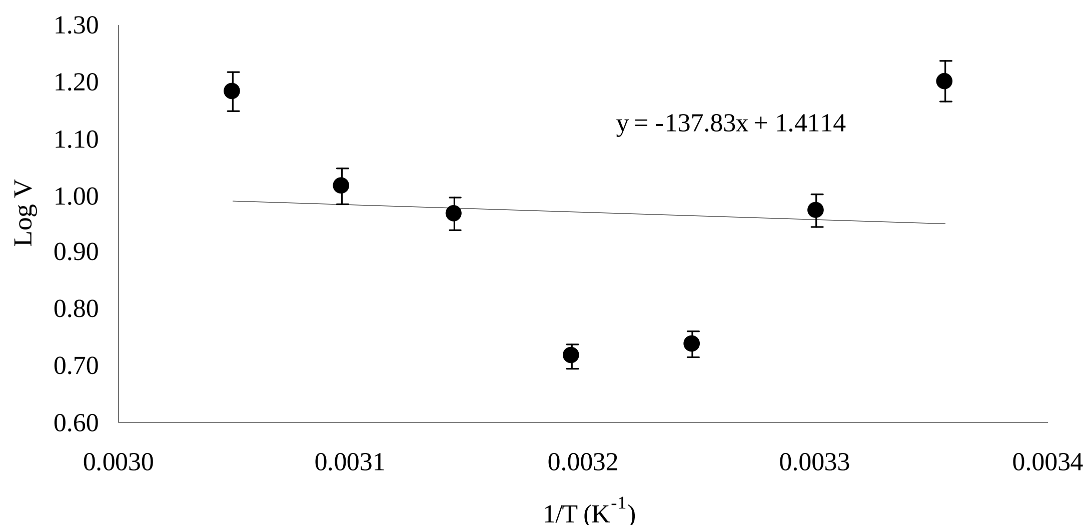


Figure (3)
Arrhenious plot for the determination of activation energy for PPO of cucumber. Mean±SD, n=3.

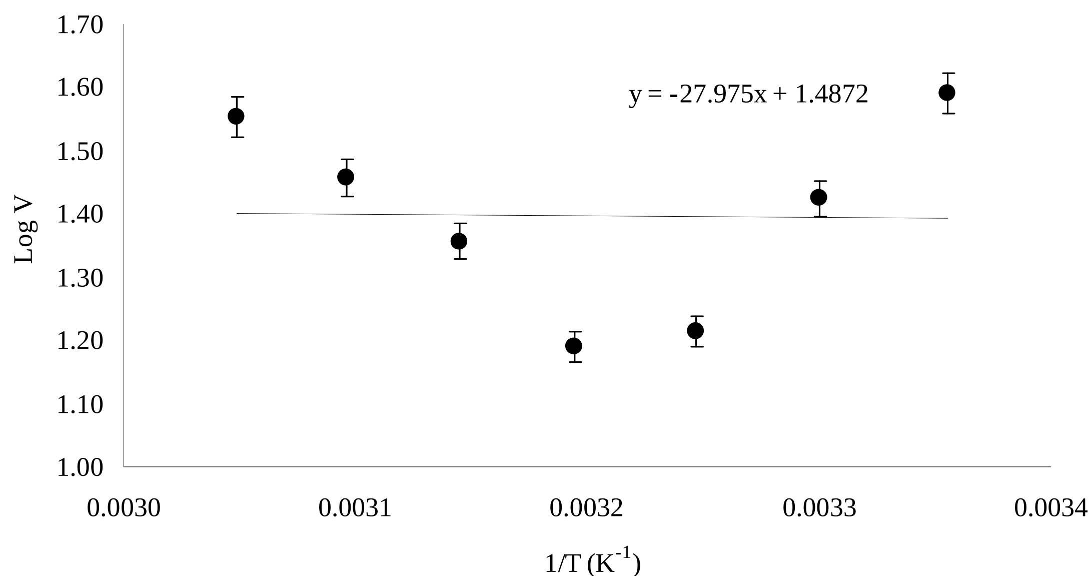


Figure (4)
Arrhenious plot for the determination of activation energy for PPO of cucurbita. Mean±SD, n=3.

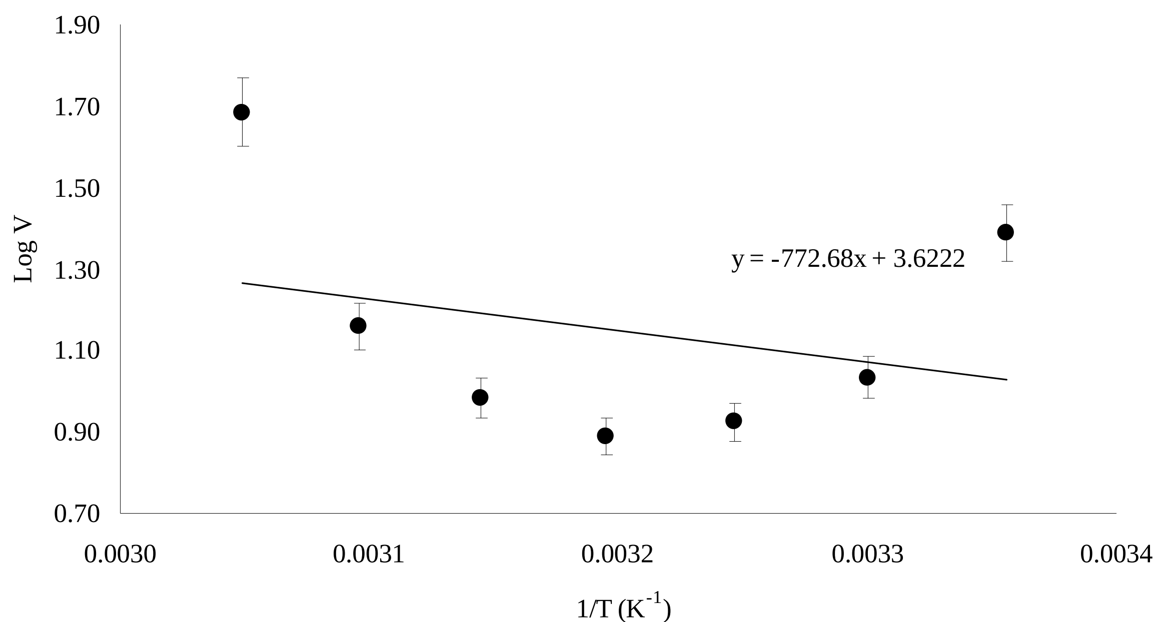


Figure (5)

Arrhenious plot for the determination of activation energy for PPO of pomegranate seed. Mean±SD, n=3.

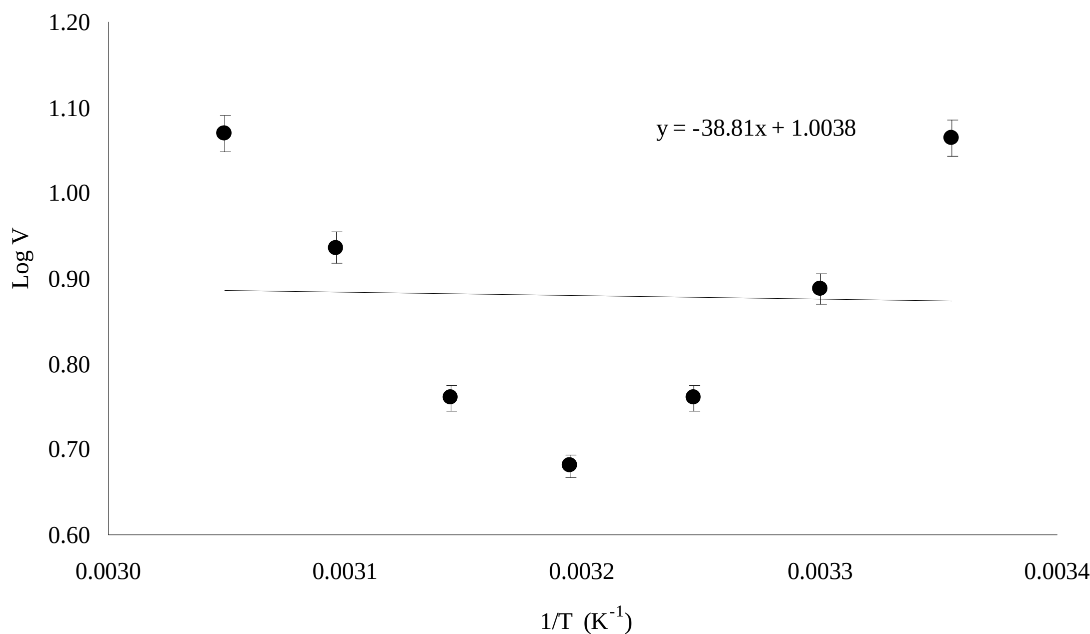


Figure (6)

Arrhenious plot for the determination of activation energy for PPO of pomegranate rind. Mean±SD, n=3.

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